

Fusiogenic Activity of Natural Amphiphiles, 5-*n*-Alkylresorcinols in a Yeast Protoplast System

Arkadiusz Kozubek^a and Jacek Skała^b

^a Institute of Biochemistry,

^b Institute of Microbiology, Wrocław University, Przybyszewskiego 63/77, 51–148 Wrocław, Poland

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Two homologues of cereal grain resorcinolic lipids, 5-*n*-heptadecylresorcinol and 5-*n*-heptadecenylresorcinol studied in the system employing yeast cell protoplasts showed marked fusiogenic activity. The frequency of hybrid formation induced by studied amphiphiles was significantly higher than that obtained with the use of 40% (w/v) polyethylene glycol 4000. The resorcinolic lipids as fusion-inducing agents did not affect regeneration of the cellular wall. The fusiogenic activity of resorcinolic lipids lost when calcium ions were absent in the medium. Fusiogenic activity of studied amphiphiles is related to their ability to induce non-bilayer structures within the cellular membranes.

Introduction

Resorcinolic lipids, the natural amphiphilic molecules isolated from cereal grains are long chain homologues of 1,3-dihydroxy-5-methylbenzene with the side chains of C₁₃ to C₂₇ in length that are saturated monoenoic and dienoic (Wenkert *et al.*, 1964; Wieringa, 1967; Kozubek *et al.*, 1979; Hengtrakul *et al.*, 1991; Mullin and Collins, 1991), all of *cis* configuration. The position of double bonds is not related to the chain length and does not differ (Kozubek and Tyman, submitted) to that determined in resorcinolic lipids present in other material, i.e., at 8 and 10 position (Tyman, 1991). Resorcinolic lipids were shown previously to be active in modification and alteration of the structure and function of biological membranes (Kozubek and Demel, 1980, 1981; Kozubek, 1984, 1985, 1987, 1987a; Kozubek *et al.*, 1988; Kozubek and Wroblewski, 1990; Kozubek *et al.*, 1992). The ³¹P-NMR data showed that incorporation of resorcinolic lipids into phospholipid bilayer induces formation of nonlamellar structures within the bilayer (Kozubek and Demel, 1981). This observation and the data indicating participation of nonlamellar structures in the fusion events (Verkleij, 1984; Frederik *et al.*, 1989; Siegel, 1993; Siegel *et al.*, 1994) suggests that the title compounds

might also act as fusiogenic agents. Our previous preliminary experiments confirmed this supposition (Kozubek, 1986), showing that 5-*n*-nonadecenylresorcinol at the concentrations of 125 μm induced fusion of human erythrocytes as a model cells. In this communication the fusiogenic effect of 5-*n*-heptadecyl and 5-*n*-heptadecenyl resorcinols (the main members of rye grain resorcinolic lipids) on two different strains of yeast and its comparison to the effect of usually used polyethylene glycol 4000 (Anne and Peberdy, 1975; Svoboda, 1978) are presented.

Materials and Methods

Resorcinolic lipid homologues studied were isolated chromatographically from rye grain extracts as described earlier (Kozubek, 1985a) and used as stock ethanolic 50 mm solution.

Yeast strains, *Saccharomyces cerevisiae* KL14-4A *a*, *his1*, *trp2*, *rho*⁺ and *Saccharomyces cerevisiae* IL126-7A *a*, *ura*, *rho*⁺ were obtained from Dr. P. Slonimski (Dujon *et al.*, 1977).

The following media were used: YPG (1% (w/v) yeast extract, 1% (w/v) peptone, 2% (w/v) glucose), G0 (0.67% (w/v) yeast nitrogen base without amino acids (Difco), 2% (w/v) glucose, 2% (w/v) agar), RM (regeneration medium) the same as G0 but with 3% (w/v) agar and 1.2 M sorbitol. If necessary G0 was supplemented with histidine, tryptophan and uracil (20 μg/ml each).

Reprint requests to Dr. A. Kozubek.
Telefax: (+71) 252930.

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Protoplast formation, fusion and regeneration was proceeded as described by Skala *et al.* (1988). Yeast strains were grown in 100 ml of YPG medium at 28 °C with shaking. The cells from exponentially growing culture were harvested, washed once with water and resuspended in 10 ml of 10 mM Tris (tris[hydroxymethyl]aminomethane)-HCl – 10 mM EDTA buffer, pH 9.3 containing β -mercaptoethanol (8 μ l/ml). The cellular suspension was incubated for 30 min at 37 °C with occasional shaking. The cells were then collected, washed once with 1.2 M sorbitol and resuspended in 10 ml of the 1.2 M sorbitol, 10 mM EDTA, 10 mM Tris-HCl buffer (pH 7.5) with Zymolyase-100T (Kirin Brewery Co., Tokyo, Japan) at concentration of 20 μ g/ml. The cells were incubated at 37 °C with gentle occasional shaking until near 100 per cent of cells were changed to protoplasts. This process was followed by microscopical inspection. Protoplasts were collected by centrifugation (300 \times *g* for 5 min), washed twice and finally suspended in 1.2 M sorbitol.

Protoplasts of two different strains were mixed in 1:1 ratio (about 2×10^9 protoplasts of each strain) and concentrated by centrifugation at 300 \times *g* for 5 min. The resulting pellet was gently suspended in 10 ml of 10 mM Tris-HCl (pH 9.3) buffer containing 10 mM CaCl₂ and 40% (w/v) polyethylene glycol (mol. wt. 4000) or in 10 ml of the same buffer but with 1.2 M sorbitol (instead of glycol) and alk(en)ylresorcinol studied. The suspension was incubated with gentle shaking at 30 °C for 30, 60 or 90 min. The glycol or remaining alk(en)ylresorcinol were removed by washing twice with 1.2 M sorbitol and protoplasts were then suspended in 10 ml of fresh 1.2 M sorbitol. For estimation of the fusion efficiency the protoplasts were mixed with melted regeneration medium at 42 °C and poured onto Petri dishes filled with the same but solidified medium. The dishes were incubated at 28 °C for 10–14 days and the number of hybrid colonies was counted.

All chemicals used were of the highest available purity.

Results and Discussion

Protoplasts of two auxotrophic yeast strains KL14-4A and IL126-7A of the same mating type were mixed in a 1:1 ratio and the fusion process

was carried out with 40% PEG or resorcinolic lipid as described in Materials and Methods. The aggregated protoplasts were suspended in regeneration medium (G0 medium supplemented with 3% agar and 1.2 M sorbitol, see Materials and Methods) and poured as a thin top layer on to identical but solid medium. In this medium, only fusion products complemented nutritionally by diploid formation between the two partners taking part in the fusion process can regenerate and grow into colonies of prototrophic phenotype. The number of colonies was taken as a measure of fusiogenic activity. No colonies appeared if intact cells or protoplasts of only one of the partners were applied; or when no PEG or resorcinolic lipid treatment was used for aggregation of protoplasts.

The results show that resorcinolic lipids are able to induce fusion of other than erythroid cells. Heptadecenylresorcinol at the concentration 125 μ M, that was proved to have no effect upon the cell growth (data not shown) after 30 min of incubation induced fusion of yeast protoplasts. The frequency of hybrid formation was in this case approximately two times lower than that observed for polyethylene glycol-induced fusion. The extension of the time of the protoplasts contact with fusiogenic agent had no effect on the number of hybrids fused by PEG but drastically increased number of hybrids in case of alkenylresorcinol (Table I). The number of hybrids observed after 60 min of protoplasts incubation with alkenylresorcinol studied was 6-times higher to that observed for 30 min of incubation time. Incubation of protoplasts with alkenylresorcinol for 90 min resulted in the decrease of the number of hybrids although the number was still 4-times higher than determined after the shortest incubation time.

Table I. The number of prototrophic hybrid colonies and its relation to fusiogenic agent and time of incubation.

Fusion inducing agent	Incubation time with fusion inducer		
	Number of prototrophic hybrid colonies per 10^9 protoplast pairs		
	30 min	60 min	90 min
PEG 4000 (40%, w/v)	2500	2500	2500
5- <i>n</i> -Heptadecenylresorcinol (125 μ M)	1200	7200	4800

According to Necas (1971, 1979) protoplasts that have not regenerated their cell walls are not able to grow and die after short time. Thus, from the high frequency of prototrophic colonies appeared we can conclude that alk(en)ylresorcinol act directly on cell membrane but remained without effect upon cell wall regeneration.

In the molecular mechanism of process of fusion both dehydration of the membrane which allows close contact between cellular membranes and the presence of calcium ions that affects structure of membrane seems to be necessary (Papahadjopoulos *et al.*, 1990, and the references therein). Resorcinolic lipids as it was shown previously (Kozubek and Demel, 1980; Kozubek, 1984; Kozubek, 1985) are lytic agents when incubated with the cells in the absence of any divalent cations. The experiments in which the yeast protoplasts were incubated with either 5-*n*-heptadecylresorcinol or 5-*n*-heptadecenylresorcinol in the absence of calcium ions in the medium show drastic decrease of the number of hybrids detected. Although the saturated homologue was more effective in this "residual" fusiogenic activity than the unsaturated one the number of hybrid cells was over 7-times lower than observed at the same concentration in previous experiment (Table II). The decrease of the fusion frequency observed for unsaturated homologue was almost 40-times. The similar decrease of the fusiogenic efficiency in the absence of calcium ions was observed also for polyethylene glycol confirming necessity of this cation for effective fusion. The decrease of the fusion efficiency observed for alk(en)ylresorcinols in the absence of divalent cation may be explained by destabilising to the membrane action of these compounds, that are not compensated by the presence of calcium ion. Alkylresorcinols that are known to exhibit lower membrane-disturbing activity were found also less active in the system used above and simultaneously can induce higher fusion, although still lower than in the presence of calcium ions. These results are in good agreement

Table II. Fusiogenic effect of resorcinolic lipids and polyethylene glycol in the absence of calcium ion in the medium. The incubation time with fusiogenic agent was 60 min. The values in the table represent the number of prototrophic hybrid colonies per 10^9 of protoplast pairs.

Fusion inducer	Concentration of fusion inducer			
	40% (w/v)	62 μ M	125 μ M	250 μ M
5- <i>n</i> -Heptadecylresorcinol	–	540	1000	480
5- <i>n</i> -Heptadecenylresorcinol	–	170	190	250
PEG 4000	800	–	–	–

to our recent data (Dabek and Kozubek, 1993) and Stasiuk and Kozubek, (submitted) indicating that divalent cations modulate the haemolytic activity of various alk(en)ylresorcinol homologues and that this modulation is both dependent on the chain length and unsaturation of the homologue.

Presented above data strongly support already accepted participation of the non-bilayer structures, regardless their cause, in the process of cellular fusion. The data also demonstrate that in some cases the effectiveness of the fusion can be improved by the use of low concentrations of amphiphilic fusiogenic lipids or compounds of known non-bilayer structure inducing properties instead of high concentrations of polyethylene glycol.

In the studies of fusiogenic properties of various agents that could be of practical meaning several model systems are employed. Liposomal and erythrocyte systems however cannot submit the information concerning the viability of obtained fused cells. The application of the yeast mutants protoplast system, in which only the cells that gained the ability to live in minimal media are estimated, showed its superiority over the other model systems for assessing the fusiogenic activity.

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